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Field Reaction to Sclerotinia Blight among Transgenic Peanut Lines Containing Antifungal Genes

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ABSTRACT

Peanut (*Arachis hypogaea* L.) is susceptible to many diseases. In the southwestern USA and other regions where peanut is grown, diseases caused by fungi are a major threat to profitable production. Transgenic peanut lines possessing fungal resistance genes offer an alternative to traditional resistance and fungicide application in managing fungal diseases. Thirty-two transgenic peanut lines containing antifungal genes (a rice chitinase and/or an alfalfa glucanase) were evaluated for their reaction to Sclerotinia blight caused by *Sclerotinia minor* Jagger in small field plots (6.1 by 7.6 m) for 3 yr. Peanut lines were arranged in a complete randomized block design with three replications. Disease incidence was recorded throughout the growing season and data were analyzed for statistical significance. Over the 3-yr period, average disease incidence for the most resistant lines—188, Southwest Runner, 416, 540, and 654—was 0.0, 1.0, 10.0, 14.0, and 16.0%, respectively. The cultivar Okrun was most susceptible with an average disease incidence of 58.0%. All other lines had varying degrees of resistance but averaged at least 15.5% less disease than Okrun over the 3-yr period. Transgenic peanut lines with partial resistance to Sclerotinia blight were identified which may be useful in traditional breeding programs for fungal resistance.

Cultivated peanut is an economically important crop throughout the world. Peanut is susceptible to many pathogens, with most damage being caused by fungi (Melouk and Backman, 1995). Soilborne fungi cause diseases that adversely affect peanut health and productivity throughout the growing areas of the USA. Diseases such as pod rot (*Rhizoctonia solani* Kühn, *Pythium myriotylum* Drechs.), crown rot (*Aspergillus niger* Tiegh.) and southern blight (*Sclerotium rolfsii* Sacc.) occur in all U.S. peanut-producing areas, while others such as Sclerotinia blight are limited to certain geographic regions. Sclerotinia blight is of major concern to peanut producers in the southwestern USA. Early symptoms of Sclerotinia blight include wilting and stem lesions with white mycelium growth. Progression of the disease can be rapid under optimal environmental conditions, which include a cool and damp plant canopy, ultimately resulting in light tan lesions on stems, stem shredding, and plant death. Depending on severity of field infestation, yield losses due to such soilborne diseases may be as high as 50% (Melouk and Backman, 1995). Traditional breeding and screening practices have resulted in cultivars resistant to fungal diseases that are suitable for commercial use (Smith et al., 1991; Simpson et al., 2000), but results have been limited. Expensive fungicide applications throughout the growing season are often required for effective disease management. Recent reductions in the U.S. peanut price support system along with these other facts have resulted in the urgent need for effective alternative methods of disease management that will provide disease control without costly inputs.

One strategy that could be used to produce disease-resistant peanut cultivars is the identification of genes responsible for fungal resistance and the use of them to genetically engineer cultivated peanut. Most fungi contain chitin, a homopolymer of β-1,4-linked N-acetylglucosamine, as a major component of their cell walls (Wessels and Seitsma, 1981; Zhang et al., 2001). All organisms that contain chitin also produce chitinases, which are hydrolases that degrade the polymer by breaking its β-1,4 linkages, presumably for morphogenesis of cell walls and exoskeletons (Kellmann et al., 1996; Kramer and Muthukrishnan, 1999). Although plants do not produce chitins, many have been shown to produce chitinases as a defense response to chitin-containing pathogens (Boller et al., 1983; Boller 1985; Mauch et al., 1988a). Recently, another hydrolase, β-1,3-glucanase, has also been suggested to be part of certain plant defense systems against fungal infection (Lin et al., 1995; Lorito et al., 1998; Lozovaya et al., 1998; Mauch et al., 1988a). Also, purified plant enzymes have been shown to hydrolyze fungal cell walls, inhibit the growth of fungal pathogens, and inhibit the induction of the chitinase promoter associated with the plant defense response (Mauch et al., 1988a, 1988b). Pathogenesis-related proteins such as chitinases and β-1,3-glucanases that are capable of hydrolyzing the cell walls of many fungi that attack plants are rational candidates for overexpression to produce disease-resistant crops.

Transgenic peanut lines that express a chitinase from rice (*Oryza sativa* L.) and or a glucanase from alfalfa (*Medicago sativa* L.) were previously produced using somatic embryos of the cultivar Okrun (Chenault et al., 2002). These peanut lines were analyzed for stable transgene inheritance and expression (Chenault et al., 2002), as well as for their response to Sclerotinia blight under greenhouse conditions (Chenault et al., 2003).

Field evaluation of peanut germplasm is a necessary step in determining the fitness for resistance to Sclerotinia blight of selected genotypes under natural environmental conditions. Coffelt and Porter (1982) conducted three field screening tests where they identified genotypes resistant to Sclerotinia blight based on morphological or physiological characteristics. Akem et al. (1992) monitored disease incidence and disease progress to

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evaluate 19 peanut genotypes for field resistance to Sclerotinia blight. Sclerotial production and viability on peanut pods in the field has also been used for determining resistance to Sclerotinia blight (Akem et al., 1997). The objectives of this study were (i) to determine the level of transgenic plant line resistance to Sclerotinia blight in the field and (ii) to evaluate the stability of that resistance over a 3-yr period.

MATERIALS AND METHODS

Transgenic peanut lines were produced from somatic embryos of the cultivar Okrun and analyzed as previously reported (Yang et al., 1998; Chenault et al., 2002). Thirty-two transgenic lines with single-copy transgene insertions were chosen for evaluation in field experiments. Field tests were conducted for three growing seasons (2000–2002) at the Oklahoma State University Agricultural Experiment Station in Caddo County, Oklahoma. Disease pressure was assessed for each test by determining the number of *S. minor* sclerotia present in soil samples taken from each plot within two weeks after planting. Viable sclerotial density was determined from the top 5 cm of field soil by a modified elutriation technique (Porter and Steele, 1983). Each individual plot was 6.1 by 7.6 m and consisted of six rows: four rows of transgenic test lines, one of the resistant cultivar Southwest Runner (Kirby et al., 1998), and one of the susceptible cultivar Okrun. Plant lines were arranged in a complete randomized block design with 8 plots per replication and 3 replications per test. All seed was treated with TOPS 90 fungicide (Gustafson, Plano, TX; 2.5 g kg^{-1} seed) before planting. Seeds were hand-planted 23 cm apart with 20 seed per row on 15 May 2000 through 2002. All plots were weeded on a weekly basis and assessed for disease symptoms. Disease incidence (number of diseased plants per line per replication) was recorded at weekly intervals after initial onset. The percentage of symptomatic plants was determined by the presence of visible above-ground symptoms. Plants were harvested individually on 15 Oct. 2000 through 2002 and returned to the laboratory for analysis. All statistical analyses were conducted using PC SAS Version 8.2 (SAS Institute, 1985). Analysis of variance techniques with PROC MIXED were performed. Since Okrun was considered as a control line, Dunnett's procedure was used by placing ADJUST = DUNNETT in an LSMEANS statement to draw comparison of each line to Okrun. Each line's comparison to Okrun depends on the line's estimated standard error. Therefore, two lines with similar means could have different conclusions drawn about them regarding their respective significance to Okrun.

RESULTS

Sclerotial density was uniform throughout the test plot area and was consistent over the 3-yr period in which field tests were conducted, averaging 3 sclerotia 100 g^{-1} soil (data not shown). In 2000, disease incidence of Sclerotinia blight among peanut lines tested ranged from 0 to 47% (Table 1) with all lines becoming symptomatic except transgenic peanut line 188. Of the transgenic peanut lines tested, 16 lines had significantly higher levels of Sclerotinia blight resistance when compared to the susceptible parent line Okrun. Disease incidence ranking of lines tested in 2000 is shown in Table 1. Of the 34 lines tested, line 188 ranked first above the resistant cultivar Southwest Runner whereas the susceptible cultivar Okrun ranked 34th.

Again in 2001, all peanut lines tested had Sclerotinia blight symptoms with the exception of transgenic peanut line 188. In 2001, disease incidence ranged from 0 to 67% (Table 1). The increased disease incidence recorded in 2001 enabled the initial identification of possible Sclerotinia blight resistant transgenic lines among those being tested. Fourteen transgenic lines had significantly more resistance to Sclerotinia blight when compared to Okrun. These same lines were also significantly more resistant to Sclerotinia blight in 2000. Disease incidence ranking of lines tested in 2001 again placed line 188 as first, while line 24 was the most susceptible, placing 34th below the susceptible cultivar Okrun (Table 1). Overall, lines were consistent in field resistance levels recorded for 2000 and 2001 with the correlation coefficient for disease incidence ranking being 0.65 ($P = 0.01$, Table 2).

As in the previous two years, all lines tested in 2002 showed symptoms of Sclerotinia blight with the exception of line 188 (Table 1). Disease incidence recorded in 2002 ranged from 0 to 65% with Okrun and line 146 having the highest percentage of disease. Seventeen transgenic lines demonstrated significantly higher levels of Sclerotinia blight resistance than the parent line Okrun, 14 of which were consistent over the 3-yr period during which tests were conducted. Disease incidence ranking for all lines tested in 2002 is shown in Table 1. Field performance correlation coefficients comparing 2001 through 2002 and 2000 through 2002 were 0.64 ($P = 0.01$) and 0.50 ($P = 0.01$), respectively (Table 2).

Disease incidence ranking was also performed solely for the 14 transgenic lines consistently demonstrating increased resistance to Sclerotinia blight over the 3-yr testing period (Table 1), since consistent field performance is necessary for a plant line to be useful in breeding programs for resistance. Disease incidence ranking was identical over the 3 yr for lines 188, Southwest Runner, and 416, placing them at positions 1, 2, and 3, respectively. Disease incidence ranking correlation coefficients (Table 2) calculated using only those lines with resistance significantly higher than Okrun were higher than those calculated for all lines tested when comparing years 2000 through 2001 and 2000 through 2002.

DISCUSSION

Testing of peanut lines for disease resistance, particularly for resistance to Sclerotinia blight, has been successful in previous studies using small field plots such as those used in this study (Akem et al., 1992; Smith et al., 1991). Such testing has resulted in the identification and eventual release of disease-resistant peanut cultivars for commercial production (Akem et al., 1992, 1997; Smith et al., 1991). Fourteen of the transgenic lines tested in this study demonstrated consistent increased resistance to Sclerotinia blight, ranging from 43 to 100% reduction in disease incidence compared to their parent line Okrun over a 3-yr period.

Line 188 had the lowest disease incidence in all 3 yr

Table 1. Disease incidence and ranking of peanut lines tested for Sclerotinia blight resistance in field plots over a 3-yr period. Disease incidence rankings for individual years are shown in parentheses.

Plant line	Transgene†	Disease incidence			
		2000	2001	2002	3-yr average
		%			
Okrun	susceptible cultivar	47 (34)	62 (31)	65 (33.5)	58
SW Runner	resistant cultivar	1 (2)***	1 (2)***	1 (2)***	1***
23	C	19 (11.5)**	42 (17.5)*	38 (12)*	33*
24	C	28 (26.5)	67 (34)	31 (8.5)	42
33	C	20 (15)*	59 (30)	53 (27)	44
34	C	20 (15)*	46 (23)	42 (27)*	36
35	C	31 (33)	54 (27.5)	57 (29.5)	47
51	C	28 (26.5)	64 (32.5)	55 (28)	49
74	C	30 (30.5)	50 (26)	52 (25.5)	44
81	C	27 (23)*	40 (15)*	38 (12)*	35*
87	C	20 (15)**	32 (9)**	43 (20)*	32**
90	C	28 (26.5)	47 (24)	45 (22)	40
133	C	30 (30.5)*	43 (19.5)*	38 (12)*	37*
135	C	27 (23)	43 (19.5)	58 (31)	43
139	C	20 (15)	37 (10)	50 (24)	35
145	C	30 (30.5)*	38 (12.5)*	40 (14.5)*	36*
146	C	22 (18)	45 (21.5)	65 (25)	43
157	C	19 (11.5)	55 (29)	42 (17.5)	38
188	C	0 (1)***	0 (1)***	0 (1)***	0***
412	C	20 (15)**	25 (6)**	42 (17.5)*	29**
416	C	8 (3)***	12 (3)***	10 (3)***	10***
423	C	23 (19.5)**	38 (12.5)*	32 (10)**	31**
461	C	13 (7)***	28 (7)**	28 (6)***	23***
487	C + G	18 (10)**	41 (16)*	30 (7)**	29**
505	G	24 (21)	45 (21.5)	57 (29.5)	43
511	C	17 (8.5)	49 (25)	45 (22)*	37
514	C	12 (26.5)	38 (32.5)	61 (22.5)	37
517	C	27 (23)	42 (17.5)	42 (17.5)*	37
531	C	23 (19.5)	54 (27.5)	45 (22)	44
535	C	28 (26.5)	64 (32.5)	52 (22.5)	48
540	C + G	11 (5)***	13 (4)***	18 (4)***	14***
542	G	17 (8.5)**	30 (8)**	40 (14.5)**	29**
561	C	30 (30.5)	38 (12.5)	31 (8.5)	33
654	C + G	9 (4)***	14 (5)***	25 (5)***	16***

* Denotes significant increase of resistance as compared to Okrun at the 0.05 probability level.
** Denotes significant increase of resistance as compared to Okrun at the 0.01 probability level.
*** Denotes significant increase of resistance as compared to Okrun at the 0.001 probability level.
† C = chitinase; G = glucanase.

tested. All of the peanut lines tested in this study have a prostrate growth habit producing a closed canopy, with the exception of lines Southwest Runner and 188 which grow erect and upright with an open canopy. Previous studies have shown that morphological resistance exists among lines with upright, bunch growth habits, probably due to the lack of a dense plant canopy that contributes to optimal disease conditions (Melouk and Backman, 1995). The location of transgene insertion for 188 has not yet been determined and thus it is assumed (but unknown) that the insertion event disrupted a gene crucial for prostrate growth habit. Line 188 contains a single copy of the rice chitinase transgene and has been previously shown to have a transgene expression level 22% above background level under nonchallenged conditions (Chenault et al., 2002). Although the transgene expression level of line 188 is well within the range of hydrolase activity reported elsewhere for plant varieties with heightened fungal resistance (Lozovaya et al., 1998), we realize the upright growth habit most likely played a major role in the total resistance observed.

Also of interest are the other top three ranking transgenic lines 416, 540, and 654. Line 416, which consistently ranked third in disease incidence over the 3-yr period, contains a single copy of the rice chitinase and has a transgene expression level of 11% above background level (Chenault et al., 2002). The disease resistance exhibited by line 416, which has a prostrate growth habit and a much lower level of transgene expression than that of 188, suggests that extremely elevated hydrolase activities may not be necessary to achieve reasonable resistance to Sclerotinia blight. These results also lend support to thoughts that the resistance reported for 188 may be partly morphological.

Lines 540 and 654 contain both the rice chitinase and the alfalfa glucanase transgenes and performed equally well in the tests, averaging a disease incidence ranking of 4.3 and 4.7, respectively. Both lines have elevated hydrolase activities with chitinase activities measuring

Table 2. Correlation coefficients† of disease incidence rankings between years tested for Sclerotinia blight resistance in field plots.

Peanut genotypes	Years		
	2000 and 2001	2001 and 2002	2000 and 2002
All (n = 34)	0.65**	0.64**	0.50**
Increased *S. minor* resistance (n = 16)	0.86***	0.69**	0.83***

** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.
† $r = 1 - [6(\Sigma d^2)]/n[(n - 1)(n + 1)]$.

22% above baseline. However, glucanase activity in either line was not significant above background level (Chenault et al., 2002). It is worth noting that line 487, which also contains both transgenes, averaged a ranking of 9.3 over the 3-yr testing period. Chitinase and glucanase activities are elevated in line 487, measuring 28% above baseline for both hydrolases (Chenault et al., 2002). These results do not support assumptions that these two transgenes would work well in concert to further boost fungal resistance (Zhu et al., 1994).

While the majority of transgenic lines with consistently higher resistance to Sclerotinia blight had a disease incidence ranking that was maintained or improved over the 3-yr test period, lines 87, 412, and 542 appeared to lose resistance over time. The same is true of several other transgenic lines tested that did not differ significantly from Okrun in their reaction to Sclerotinia blight. One possible explanation for this loss of resistance is that of transgene silencing which has been reported in other studies (Iyer et al., 2000; Kunz et al., 2001), but which appears to be more common among transformed plants containing multiple copies of the transgene(s).

In this study, the measurement of percentage of disease incidence and disease incidence ranking over a 3-yr period enabled the identification of transgenic peanut lines that consistently demonstrated a stable level of resistance to Sclerotinia blight under field conditions. The results from this study are in agreement with those from a previous study in which these same peanut lines were tested for Sclerotinia blight resistance under greenhouse conditions (Chenault et al., 2003). This agreement was expected based on other reports using the same series of methods to test for Sclerotinia blight resistance (Akem et al., 1992, 1997; Goldman et al., 1995).

The levels of hydrolase activity observed in these transgenic peanut lines (0–37%; Chenault et al., 2002) is comparable to that recorded for plant varieties with elevated fungal resistance. Lozovaya et al. (1998) reported that β-1,3-glucanase activity levels were 33% higher in maize genotypes resistant to *A. flavus* infection than in those considered susceptible. Similar observations were reported by Neucere et al. (1995) when examining glucanase levels in mature kernels of *A. flavus* susceptible and resistant genotypes. Others have been successful in generating transgenic plants with resistance to fungal infection through the introduction of a foreign chitinase gene (Broglie et al., 1991; Lin et al., 1995; Lorito et al., 1998). Broglie et al. (1991), reported an increased resistance to *R. solani* infection in the roots of tobacco containing a chitinase transgene.

The identification of transgenic peanut lines with enhanced fungal resistance under field conditions has provided necessary information for determining the usefulness of such plant lines as parents in traditional breeding programs for improving resistance to Sclerotinia blight.

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